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Properties of an NAD(H)-containing methanol dehydrogenase and its activator protein from *Bacillus methanolicus*

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Oxidation of C_1-C_4 primary alcohols in thermotolerant *Bacillus methanolicus* strains is catalyzed by an NAD-dependent methanol dehydrogenase (MDH), composed of ten identical 43000-M_r subunits. Each MDH subunit contains a tightly, but non-covalently, bound NAD(H) molecule, in addition to 1 Zn^{2+} and 1–2 Mg^{2+} ions. The NAD(H) cofactor is oxidized and reduced by formaldehyde and methanol, respectively, while it remains bound to the enzyme. Incubation of MDH with methanol and exogenous NAD (coenzyme) results in reduction of this NAD coenzyme. Both NAD species are not exchanged during catalysis. NAD thus plays two different and important roles in the MDH-catalyzed reaction, with the bound NAD cofactor acting as primary electron acceptor and the NAD coenzyme being responsible for reoxidation of the reduced cofactor. MDH obeys a ping-pong type reaction mechanism, which is consistent with such a temporary parking of reducing equivalents at the MDH-bound cofactor. Spectral studies show that, in the presence of exogenous NAD and Mg^{2+} ions, MDH interacts with a previously identified 50000-M_r activator protein. The activator protein appears to facilitate the oxidation of the reduced NADH cofactor of MDH, which results in a strongly increased turnover rate of MDH.

Keywords: methanol dehydrogenase; NAD; activator protein; *Bacillus methanolicus*.

The initial oxidation of methanol to formaldehyde in methanol-utilizing microorganisms is catalyzed by at least three different types of enzymes. Yeasts employ a peroxisomal alcohol oxidase enzyme that contains FAD and catalyzes the oxidation of methanol into formaldehyde and hydrogen peroxide, using oxygen as the electron acceptor (Harder and Veenhuis, 1989). Gram-negative bacteria oxidize methanol with a methanol dehydrogenase that is located in the periplasmic space and which contains pyrroloquinoline quinone (Anthony, 1986; Duine et al., 1986). Gram-positive methylotrophic bacteria only recently have been identified and studied in more detail. These organisms, e.g. *Amycolatopsis methanolica* (Kato et al., 1974; De Boer et al., 1990), *Mycobacterium gastri* (Kato et al., 1988) and *Bacillus methanolicus* (Arfman et al., 1989), lack a periplasmic space and do not possess a classical pyrroloquinoline-quinone-containing methanol dehydrogenase. Evidence is accumulating that these gram-positive bacteria employ a different type of NAD-dependent alcohol dehydrogenase during growth with methanol and other short-chain primary alcohols (Bystrykh et al., 1993a).

The presence of a cytoplasmic, NAD-dependent methanol dehydrogenase (MDH) was demonstrated in *B. methanolicus* and all other thermotolerant, methanol-utilizing *Bacillus* spp. investigated (Arfman et al., 1989). The enzyme consists of ten identical subunits of M_r 43000, arranged in a sandwich of two pentagonal rings; each subunit contains 1 Zn^{2+} and 1–2 Mg^{2+} ions (Vonck et al., 1991). Zinc is commonly found in the active site of alcohol dehydrogenases, but the presence of magnesium had not been reported before. Studies with purified proteins showed that MDH activity with C_1-C_4 primary alcohols and its affinity for (exogenous) NAD and alcohol substrates were strongly increased by a soluble 50000-M_r protein (activator protein) of *B. methanolicus*. At physiological methanol concentrations (0.1–1 mM), the methanol turnover rate of MDH *in vitro* was increased up to 40-fold by the activator protein. This activation process strictly required exogenous Mg^{2+} (Arfman et al., 1991). Synthesis of MDH and activator protein in *B. methanolicus* was regulated coordinately (Arfman et al., 1992).

Although *B. methanolicus* MDH is capable of oxidizing C_1-C_4 primary alcohols and requires NAD as a coenzyme, it does not belong to the large family of zinc-containing long-chain, NAD-dependent alcohol dehydrogenases (ADH) exemplified by horse liver ADH and *B. stearothermophilus* ADH, as based on N-terminal amino acid sequence analysis (Jörnvall et al., 1987). In contrast, *B. methanolicus* MDH showed substantial sequence similarity with members of a different family of alcohol dehydrogenases (family III) found in various organisms, e.g. *Zymomonas mobilis* (ADH2), *Saccharomyces cerevisiae* (ADH4), *Clostridium acetobutylicum*, and *Escherichia coli* (De Vries et al., 1992; Vonck et al., 1991; Conway et al., 1987; Williamson and Paquin, 1987; Youngleson et al., 1989; Conway and Ingram, 1989; Reid and Fewson, 1994). The latter enzymes are generally a lot smaller (dimers or tetramers), do not oxidize methanol, and contain iron or zinc; no magnesium has been reported. Related decameric methanol dehydrogenases containing magnesium, however, subsequently were detected in other gram-positive methanol-utilizing bacteria, the actinomycetes *A. methanolica* and *M. gastri* (Bystrykh et al., 1993a–c). There is physiological and mutant evidence now showing that the decameric proteins in these bacteria are functional during growth with primary alco-

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Abbreviations. MDH, NAD-dependent methanol dehydrogenase; ADH, NAD-dependent alcohol dehydrogenase.

Enzymes. Methanol dehydrogenase (NAD-dependent) (EC 1.1.1.244); alcohol oxidase (EC 1.1.3.13).

hols, which catalyzes the initial step in their oxidation (Arfman et al., 1992; Brooke et al., 1989; Hektor and Dijkhuizen, 1996). These decameric alcohol dehydrogenases also have been detected in related nonmethylotrophic gram-positive species (e.g. in various *Rhodococcus* and *Desulfovibrio* species) which suggests that they may be a lot more widespread in the microbial world (Van Ophem et al., 1991; Nagy et al., 1995; Hensgens et al., 1993, 1995).

The remarkable structural and kinetic characteristics of MDH and the unknown mechanism of its activation encouraged us to study the properties of the MDH and activator proteins of *B. methanolicus* in more detail.

EXPERIMENTAL PROCEDURES

Purification of MDH and activator protein. MDH and the activator protein were isolated from *B. methanolicus* cells grown in a methanol-limited chemostat culture at $D = 0.1\ h^{-1}$. The preparation of crude extract and the purification of MDH and activator protein to homogeneity have been described previously (Arfman et al., 1989, 1991; Vonck et al., 1991). Prior to use, the MDH and activator protein preparations were desalted on a Bio-Rad PD-10 column, equilibrated with 100 mM glycine/KOH, pH 9.5, without Mg^{2+}. For some experiments, only partially purified activator protein preparations were used, obtained after a hydrophobic interaction chromatography and two anion-exchange chromatography steps, which correspond to purification steps 2–4 described by Arfman et al. (1991). The final preparation contained approximately 300 activator units/mg protein (3.36 nmol activator subunits), and was completely devoid of MDH activity. MDH and activator protein concentrations are expressed in MDH subunits (M_r 43000) and activator subunits (M_r 27000), respectively (Arfman et al., 1989, 1991).

Enzyme assays. Spectrophotometric assays were performed with a Hitachi model 100–60 spectrophotometer. Unless stated otherwise, enzyme assays were performed at 50°C, using prewarmed buffer solutions. NAD-dependent MDH activity, dye-linked MDH activity (phenazine methosulfate/2,6-dichloroindophenol) and stimulating activity of the activator protein were measured as described earlier (Arfman et al., 1989, 1991). Alcohol-dependent reduction of *p*-nitroso-*N*,*N*-dimethylaniline (ε_{440} = 35.4 $mM^{-1} \cdot cm^{-1}$) was measured with methanol or ethanol as a substrate, using the assay system described by Dunn and Bernhard (1971). 1 U MDH enzyme activity is defined as the amount of protein catalyzing the conversion of 1 μmol substrate/min under the assay conditions described. 1 U stimulating activity of the activator protein is defined as the affinity constant of the MDH activation reaction (as determined from titration curves) and corresponds to 0.28 μg purified protein (purified activator: 3600 U/mg protein; Arfman et al., 1991).

Absorption coefficients of MDH and activator protein. The specific absorption coefficients of MDH and activator protein at 280 nm were determined from the absorbance at 205 nm and 280 nm using the chromatographic method of Van Iersel et al. (1985). Purified MDH (0.7 nmol) was injected on a Superose 12 gel-filtration column equilibrated with 100 mM glycine/KOH, pH 9.5 (buffer A), plus 5 mM $MgSO_4$, equilibrated at a flow rate of 0.5 ml/min (at room temperature). Purified activator protein (1.24 nmol) was separately injected on a Superose 12 column equilibrated with 100 mM potassium phosphate, pH 6.5, containing 0.1 M NaCl (buffer B) at 0.5 ml/min. The absorption spectra of native MDH (decameric), MDH subunits (Arfman et al., 1991), and activator protein in the eluent were measured with an on-line Hewlett Packard 1040A photodiode array spectrophotometer.

Spectrophotometry. Ultraviolet/visible absorption spectra were measured with a Hewlett Packard 8452 A photodiode array spectrophotometer, equipped with a stirring module. Difference spectra of MDH were recorded at 50°C. Redox activity of the MDH-bound chromophore was demonstrated by analyzing difference spectra of MDH (9.9 μM), dissolved in buffer A plus 5 mM $MgSO_4$, recorded after stepwise additions of formaldehyde (10–60 μM) and methanol (110 mM). Since formaldehyde reacts with (residual) 2-mercaptoethanol in the preparation, the spectra were corrected for the formation of this product, assuming that the absorbance above 400 nm is solely due to this reaction. Effects of activator protein, NAD, and Mg^{2+} on the MDH spectrum were studied by recording difference spectra after successive additions of activator protein (200 nM), NAD (25 μM) and $MgSO_4$ (5 mM) to MDH (6.5 μM) dissolved in buffer A. After activation had occurred, the mixture was separated on a Superose 12 gel-filtration column, equilibrated with buffer B at 0.5 ml/min, and spectra of eluting peak fractions were analyzed.

Fluorescence spectra were recorded at 50°C with a Perkin-Elmer spectrophotometer, type LS 50. Measurements were performed in a quartz cuvette (10-mm width) in a total volume of 3 ml. Fluorescence emission spectra of MDH were scanned in the range 360–500 nm (band width 15 nm), using a 5.3 μM MDH solution in buffer A, which was excited at 340 nm (band width 5 nm). A 30 nM solution of NADH in the same buffer served as standard. Effects of activator protein, Mg^{2+}, and NAD on the fluorescent properties of MDH-bound NAD(H) were studied by analysis of excitation spectra of MDH. Excitation spectra were scanned in the range 300–390 nm at a fixed emission wavelength of 410 nm. The complete reaction mixture in 3 ml buffer A contained: MDH, 15.9 nmol; activator protein, 0.34 nmol; $MgSO_4$, 15 μmol, and NAD, 1.5 μmol. All spectra were corrected against buffer A. Differential spectra were obtained using FL Data Manager (Perkin-Elmer) installed on an IBM-type personal computer.

Extraction of MDH-bound chromophore and denaturing gel filtration. The enzyme-bound chromophore was extracted by denaturation with urea. Acidic denaturation, such as trichloroacetic acid extraction, was avoided as it leads to degradation of NADH (Kato et al., 1986). Urea (final concentration 6 M) was added to 6.5 μM purified MDH in 1 ml 0.1 M Tris/HCl, pH 7.5, containing 5 mM $MgSO_4$. After boiling for 2 min, the preparation was applied to a Superose 12 gel-filtration column, equilibrated with buffer B containing 6 M urea, at 0.5 ml/min. As a control, 1 ml 1 mM NADH solution previously boiled with 6 M urea, was analyzed separately. Spectra of eluting peak fractions were recorded.

Analysis of the chromophore. The extracted chromophore was separated from the protein fraction by desalting 2.5 ml urea treated MDH (containing 320 nmol MDH) against 10 mM Tris/HCl, pH 8.0, containing 6 M urea (buffer C), on a Pharmacia PD-10 column. Samples (200 μl) of the 5-ml salt fraction, containing the chromophore, were applied to a Mono-Q ion-exchange column equilibrated with buffer C at a flow rate of 1 ml/min. Samples (200 μl) of urea-treated NAD, NADH, and NADPH, 10 nmol of each, served as standards. Bound material was eluted with a 0–0.9 M KCl gradient in 15 ml buffer C.

Biological activity of MDH-derived cofactor. Biological activity of the extracted cofactor was tested in a NADH-dependent acetaldehyde reductase assay system using horse liver ADH. This enzyme retained considerable activity in assay mixtures containing up to 4 M urea. Acetaldehyde reductase activity was measured by following the oxidation of NADH at 340 nm. The assay mixture (1 ml) contained the following: 0.54 ml buffer A; 10 μl horse liver ADH (1 μg); 0.45 ml 300 μM NADH solution in 10 mM Tris/HCl, pH 8.0, containing 6 M urea or

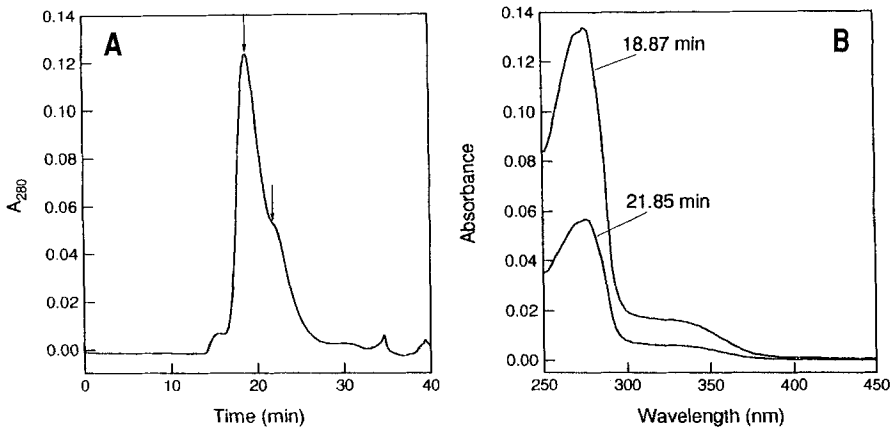

Fig. 1. Gel filtration and spectral analysis of purified MDH. Superose 12 gel-filtration chromatography of 0.7 nmol purified MDH (A). As buffer, 100 mM potassium phosphate, pH 6.5, plus 100 mM NaCl was used, with a flow rate of 0.5 ml/min. (B) Absorption spectra of native (decameric) MDH (eluting at 18.87 min) and MDH subunits (eluting at 21.85 min). Arrows indicate the retention times at which spectra were recorded.

0.45 ml low-molecular-mass fraction from the PD-10 column (see above), containing MDH-derived cofactor plus 6 M urea. After equilibration at 37 °C for 5 min, the reaction was started with 10 μM acetaldehyde.

NADH-binding experiments. The NADH-binding capacity of activator protein was determined by fluorometric titration of purified activator protein with NADH, based on the increased fluorescence of bound NADH compared to free NADH. Fixed quantities of NADH (0.3 nmol) were added to 3 nmol activator protein in 3 ml buffer A, containing 5 mM $MgSO_4$. After each addition, the fluorescence was recorded (excitation at 340 nm and emission at 455 nm).

Studies with ^{32}P-labelled NAD. The possible exchange of bound and free NAD(H) was studied by incubating MDH with [adenylate-^{32}P]NAD in standard MDH assays (A_{340}). The complete reaction mixture (2.5 ml) in buffer A contained the following: $MgSO_4$, 12.5 μmol; NAD, 25 nmol; [adenylate-^{32}P]NAD (1000 Ci/mmol), 8.3 pmol (8.3 μCi); MDH subunits, 20 nmol; partially purified activator protein, 2.8 nmol; methanol, 1.25 mmol. Control experiments were carried out with incomplete reaction mixtures (Table 1). The mixture was incubated for 10 min at 50 °C; for experiment I, the temperature was 20 °C. Spectrophotometric analysis of the reaction mixture showed that at 50 °C, 10 min was sufficient to establish equilibrium. In a control experiment, labelled NAD was incubated with baker's yeast ADH. The procedure described above was adjusted as follows: baker's yeast ADHI (subunit M_r 40000) instead of MDH, 25 nmol; ethanol instead of methanol, 420 μmol. The mixture was incubated at 30 °C. After incubation, the reaction mixtures (2.5 ml) were desalted on a Pharmacia PD-10 gel-filtration column, previously equilibrated with buffer A plus 5 mM $MgSO_4$. The protein fraction was eluted with 3.5 ml buffer A and the salt fraction in an additional 5 ml. Radioactivity present in the protein and salt fractions was measured as Cerenkov radiation in a Packard 2000 CA liquid scintillation counter (window 0–19 keV).

Coenzyme NAD requirement for MDH activity. Methanol dehydrogenase activity of purified MDH, both in the presence or absence of activator protein and exogenous NAD, was investigated in a dye linked assay containing phenazine methosulfate as (artificial) electron acceptor in combination with 2,6-dichloroindophenol. Methanol-dependent 2,6-dichloroindophenol reduction (at 50 °C) was measured at 600 nm in a reaction mixture (1 ml) containing: glycine/KOH, pH 9.5, 100 μmol; $MgSO_4$, 5 μmol; MDH, 25 pmol; activator, 50 pmol; 2,6-dichloroindophenol, 80 nmol; phenazine methosulfate, 1.25 μmol. The assays were also performed in 50 mM potassium phosphate, pH 8.0, containing 5 mM $MgSO_4$.

Steady-state kinetics. Kinetic experiments were performed by measuring initial MDH reaction rates under the same experimental conditions as used for activity measurements. For isotope studies with deuterated substrate, reactions were started with 50 mM CD_3OD.

Metal analysis. The metal composition of purified activator protein was determined with a Perkin Elmer 5100 oven atomic absorption spectrophotometer. The activator protein preparation was dialyzed extensively against 10 mM Tris/HCl, pH 7.0, containing 1 mM EDTA and subsequently against the same buffer without EDTA. The final activator protein concentration was 0.41 μM. The following elements were analyzed in duplicate: zinc, magnesium, iron, and copper.

Protein determination. Protein concentrations of the purified MDH and activator preparations were determined by measuring the absorbance at 280 nm, using the specific absorption coefficients determined as described above. Protein concentrations of partially purified preparations were determined by means of the Bio-Rad protein assay with bovine serum albumin as the standard.

Materials. All chemicals were reagent grade. Protein dye reagent and bovine serum albumin were from Bio-Rad. NAD, NADH, NADPH, baker's yeast ADHI, and horse liver ADH were from Boehringer. [Adenylate-^{32}P]NAD was purchased from Amersham. CD_3OD was from Merck.

RESULTS

Spectral analysis, NADH binding, and metal composition of purified activator protein. The absorption spectrum of activator protein revealed a single absorption maximum at 278 nm. The specific absorption coefficient of the activator protein was determined as 18600 $M^{-1} \cdot cm^{-1}$ (A_{280} 0.69 at 1 mg/ml).

The capacity of purified activator protein to bind exogenous NADH was studied in fluorometric NADH-titration experiments. This revealed binding of 1.0 ± 0.1 mol NADH/mol subunits, i.e. each activator protein subunit can bind one molecule of NADH.

Metal analysis revealed 0.43 μM zinc and 0.45 μM magnesium in a solution containing 0.41 μM activator protein subunits. Consequently, each activator protein subunit contains one zinc and one magnesium ion. Iron and copper were not detected.

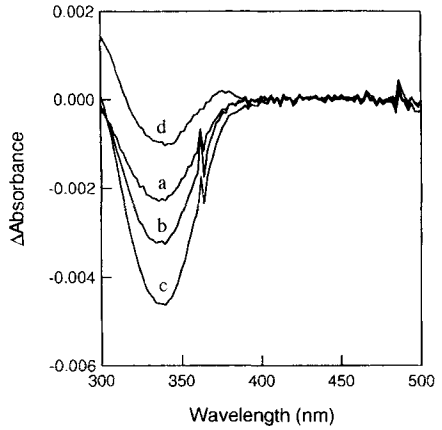

Fig. 2. Oxidation and reduction of MDH-bound chromophore. Difference absorption spectra of MDH. The reaction mixture contained 100 mM glycine/KOH, pH 9.5, plus 5 mM $MgSO_4$ and 9.9 μM MDH subunits. To the mixture, formaldehyde was added to final concentrations of 10 μM (curve a), 30 μM (curve b), and 60 μM (curve c). Subsequently, methanol was added to a final concentration of 110 mM (curve d).

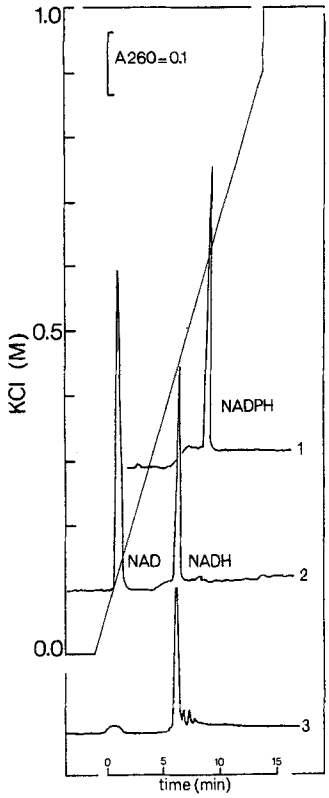

Fig. 4. Ion-exchange chromatography of NAD, NADH and NADPH. The detailed conditions are given in the Experimental Procedures section. (1) Authentic NADPH (10 nmol). (2) A mixture of authentic NAD and NADH (10 nmol each). (3) Low-molecular-mass fraction of urea-extracted MDH.

Spectral analysis of purified MDH protein. During gel filtration, the purified native MDH protein partially dissociates into its subunits (Arfman et al., 1989, 1991). The absorption spectra of the decameric form, which elutes at 18.87 min, and the monomeric form, which elutes at 21.85 min, are virtually identical (Fig. 1). Apart from a typical protein absorption peak at 276 nm, the spectrum displayed a small shoulder around 260 nm and a broad shoulder in the 300–350-nm region, which indicates the presence of a chromophore other than aromatic amino acids. The specific absorption coefficient at 280 nm of MDH was determined as 18500 $M^{-1} \cdot cm^{-1}$ (A_{280} 0.43 at 1 mg/ml), both for the native decameric protein and its subunits. This low absorption coefficient is due to the absence of tryptophan residues in the protein (De Vries et al., 1992).

Redox activity of MDH-bound chromophore. A possible role of the MDH-bound chromophore in catalysis was investigated by monitoring the effects of formaldehyde and methanol on difference (Δ) absorption spectra of the protein. Addition of a stoichiometric amount of formaldehyde to MDH (10 μM formaldehyde/9.9 μM MDH) resulted in a significant decrease of absorbance in the 340-nm region, indicative of the oxidation of the enzyme-bound chromophore (Fig. 2). A further decrease of A_{340} occurred when raising the formaldehyde concentration to 30 μM and 60 μM. The dip in absorbance at 340 nm almost completely disappeared after the addition of an excess amount of methanol (110 mM) to the reaction mixture, which shows that the enzyme-bound chromophore was reduced again by methanol. The reversible oxidation and reduction of the enzyme-bound chromophore (cofactor) indicated that it participates in the transfer of reducing

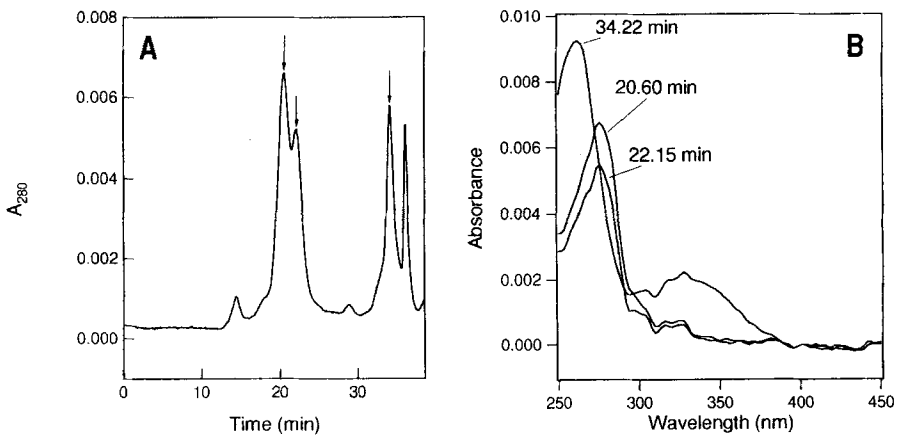

Fig. 3. Denaturating gel-filtration chromatography of MDH and spectral analysis. Urea-treated MDH (6.5 nmol) was applied to a Superose 12 gel-filtration column, equilibrated with buffer B containing 6 M urea, at a flow rate of 0.5 ml/min (A). (B) Absorption spectra of the protein peaks eluting at 20.60 min and 22.15 min and of the low-molecular-mass peak eluting at 34.22 min. The peak at 36.5 min corresponds to 2-mercaptoethanol. Arrows indicate the retention times at which spectra were recorded.

Table 1. [^{32}P]NAD labelling experiments. Recovery of label in the combined protein and salt fractions after gel filtration was greater than 95% in all experiments. 1 μCi label corresponded to 509000 cpm. The experiments were performed in duplicate. For the protein fraction measurements, the reaction mixtures were desalted after incubation; the label in the remaining protein fractions represents the degree of NAD exchange. Baker's yeast ADHI was used for experiment II. 2.8 nmol activator protein was included in experiment V.

Experiment no.	Incubation conditions		[^{32}P]NAD substrate		Protein fraction			Fraction of subunits labelled
	subunits	NAD			label	NAD	corr.	
	nmol		nCi/nmol		nCi	nmol		%
I (control)	–	25	330	–	150	0.5	–	–
II	25 ADH	25	330	ethanol	170	0.5	0	0
III	20 MDH	25	330	–	750	2.3	1.8	9
IV	20 MDH	25	330	methanol	820	2.5	2.0	10
V	20 MDH	25	330	methanol	560	1.7	1.2	6

equivalents during alcohol dehydrogenase and aldehyde reductase reactions catalyzed by MDH.

Dissociation of MDH-bound cofactor. Ammonium sulfate precipitation and exhaustive dialysis did not result in dissociation of MDH-bound cofactor. Urea treatment of MDH and subsequent (denaturating) gel-filtration chromatography could completely separate the bound cofactor from the purified enzyme (Fig. 3A). Spectral analysis showed that the protein fractions eluting at 20.60 min and 22.15 min (representing different subunit oligomeric states of MDH with identical absorption spectra) no longer displayed the characteristic absorbance around 340 nm (Fig. 3B). Instead, this absorbance now appeared in the low-molecular-mass (salt) fraction eluting at 34.22 min (Fig. 3B). The urea-extracted MDH cofactor and an urea-treated commercial NADH preparation eluted from the gel-filtration column at virtually identical retention times (34.22 min and 34.37 min, respectively). The absorption spectrum of the dissociated cofactor revealed maxima at 260 nm and 330–340 nm, which is characteristic of NADH (Fig. 3B). On the basis of the extraction conditions, it was concluded that the cofactor interacts non-covalently with the enzyme protein.

Identification and quantification of MDH-bound cofactor. The extracted cofactor was further analyzed by ion-exchange chromatography, using conditions resulting in clear separation of NAD, NADH, and NADPH (Fig. 4). The MDH-derived cofactor preparation showed a main peak that corresponded to the position of NADH and a small peak corresponding to NAD. The identity of the extracted cofactor preparation was verified in a standard horse liver ADH assay, where it could replace NADH. The amount of NADH per MDH subunit was determined by measurement of the A_{340} (ε_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$) of urea-extracted MDH preparations. This yielded values of 0.42–0.95 mol NADH/mol subunit in several separate experiments, which indicates that MDH most probably contains 1 NAD(H) cofactor molecule/subunit.

Coenzyme NAD requirement for MDH activity. MDH displayed only methanol-dependent 2,6-dichloroindophenol reduction in the presence of phenazine methosulfate following addition of exogenous NAD. This was observed at pH 8.0 and pH 9.5, both in the presence or absence of the activator protein. Catalytic amounts of NAD [K_m(NAD) = 5–10 μM] were already sufficient to support maximal activity in these NAD regenerating assay systems. These observations indicate that coenzyme NAD is required for MDH activity and that the MDH-bound NADH cofactor can be oxidized by the NAD coenzyme.

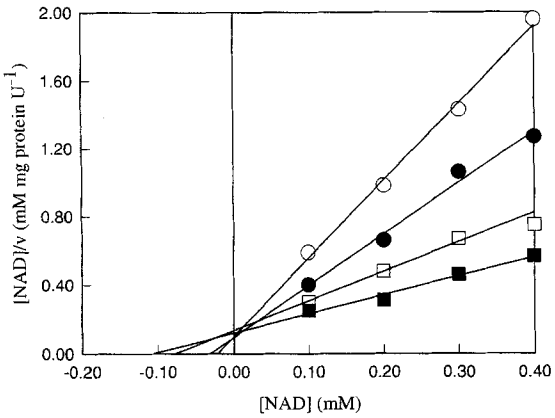

Fig. 5. Steady-state kinetics of MDH. NAD-titration plots of MDH reactions. The MDH protein concentration was 25 nM. The following methanol concentrations (mM) were applied: (○) 62.5; (●) 125; (□) 250; (■) 500.

Whereas the MDH-bound NADH cofactor cannot be oxidized directly by phenazine methosulfate/2,6-dichloroindophenol, the NADH cofactor extracted from MDH could readily be oxidized by phenazine methosulfate.

^{32}P-NAD labelling studies with MDH. The data presented above indicated that MDH employed both enzyme-bound NAD(H) (cofactor) as well as exogenous NAD (as coenzyme) for activity. We subsequently investigated whether MDH-bound NAD(H) and coenzyme NAD were exchanged during the catalytic reaction by incubating MDH with trace labelled [^{32}P]NAD and methanol. Once the reaction had reached equilibrium (based on A_{340} measurements), the protein and salt fractions were separated by gel filtration and analyzed for radioactivity. The results of these labelling experiments are presented in Table 1.

Control experiment I (absence of MDH and alcohol) showed that 2% (150 nCi) added labelled NAD (8.3 μCi) appeared in the protein fraction, which reflects the separation efficiency of the gel-filtration procedure used. Control experiment II, with baker's yeast ADHI and ethanol, showed that the ADHI protein fraction after gel filtration and correction for control experiment I, did not retain any additional label. Incubation of MDH with labelled NAD, in the absence of methanol (experiment III), resulted in a (non-dissociable) binding of 1.8 nmol NAD by 20 nmol MDH subunits, which corresponds to a degree of labelling of 9%. Addition of methanol to the incubation mixture (experiment IV) did not result in a significantly increased labelling of the MDH protein fraction. Thus, at a stage where the metha-

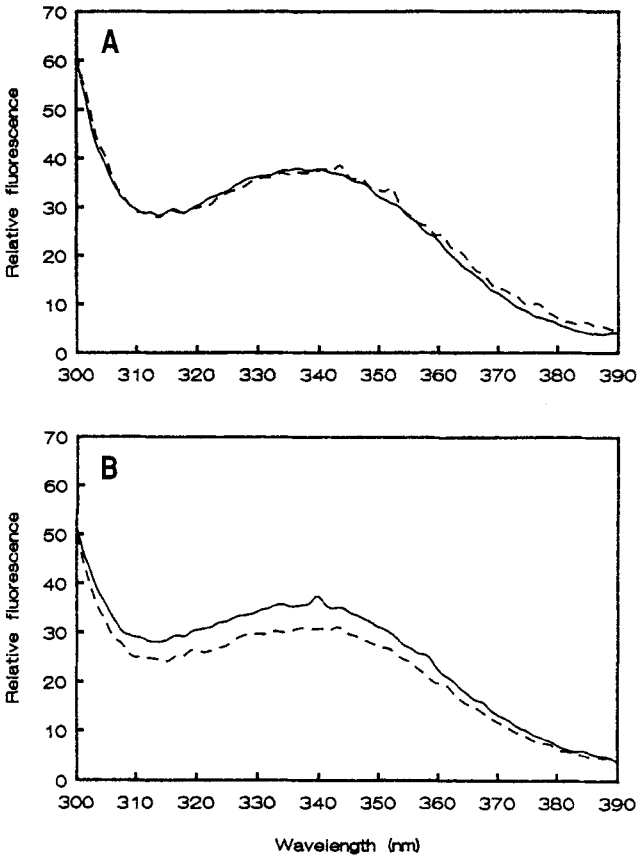

Fig. 6. Fluorescence spectrophotometry of purified MDH. Fluorescence spectra were recorded after equilibration of the samples at 50 °C. (A) Excitation spectra (emission at 410 nm) of MDH plus activator protein, in the presence (——) or absence (– – –) of Mg^{2+}. These spectra were also observed with mixtures of MDH plus NAD (without activator protein), in the presence or absence of Mg^{2+}. (B) Excitation spectra (emission at 410 nm) of a mixture containing MDH, activator protein, and NAD, in the presence (——) or absence (– – –) of Mg^{2+}.

nol dehydrogenase reaction had reached equilibrium (> 30 catalytic cycles/subunit), the presence of methanol did not significantly stimulate the appearance of [^{32}P]NAD label in MDH subunits (at most 1% stimulation). Studies in the presence of activator protein gave similar results (experiment V). These data clearly show that methanol oxidation by MDH does not involve exchange of exogenous NAD and MDH-bound NAD(H) cofactor.

Steady-state kinetics of the MDH reaction. Steady-state MDH reaction rates with varying NAD concentrations at four different methanol concentrations revealed lines crossing on the ordinate in a plot of *s/v* versus *s* (Fig. 5). MDH thus obeys a ping-pong type mechanism (Wong, 1975).

Isotope studies with deuterated methanol (CD_3OD) were used to probe for the rate-limiting step. The isotope effect for the methanol oxidation reaction, defined as the ratio $V_{max}(CH_3OH)/V_{max}(CD_3OD)$, was 1.5–2, both with and without activator protein.

MDH was not capable of catalyzing methanol- or ethanol-dependent reduction of *p*-nitroso-*N*,*N*-dimethylaniline, both with and without exogenous NAD(H) (pH range 6.5–9).

Effects of activator protein, Mg^{2+} and NAD on the spectral properties of MDH. The fluorescence emission spectrum of purified MDH, at a fixed excitation wavelength of 340 nm, displayed an optimum at 400–410 nm. In contrast, pure NADH showed a maximum at 455 nm (data not shown). The fluorescence excitation spectrum of MDH, at a fixed emission wavelength of 410 nm, showed a peak at 340 nm (Fig. 6A). Addition of activator protein, NAD, and Mg^{2+} to the MDH solution, previously equilibrated at 50 °C, significantly increased the fluorescence of MDH (Fig. 6B), which suggests a change in the environment of the bound NADH cofactor. The increased fluorescence was strictly dependent on the presence of Mg^{2+} ions, activator protein, and NAD, which indicated the formation of an activator protein · MDH · NAD · Mg^{2+} complex. This effect was further studied by absorption spectrophotometry. Stepwise addition of activator, NAD, and Mg^{2+}, respectively, to a solution containing purified MDH revealed a difference absorption spectrum characteristic for NADH (Fig. 7). This difference spectrum, with peaks at 260 nm and 340 nm, appeared only after the addition of Mg^{2+}. Addition of Mg^{2+} to a pure NADH solution did not reveal such a spectral change. Gel-filtration chromatography of the activated MDH preparation did not indicate the presence of (free) NADH in the low-molecular-mass fractions. Therefore, the altered spectral properties (fluorescence and absorbance) of MDH are due to a conformational change of the enzyme caused by the Mg^{2+}-dependent formation of a MDH · activator · NAD · Mg^{2+} complex.

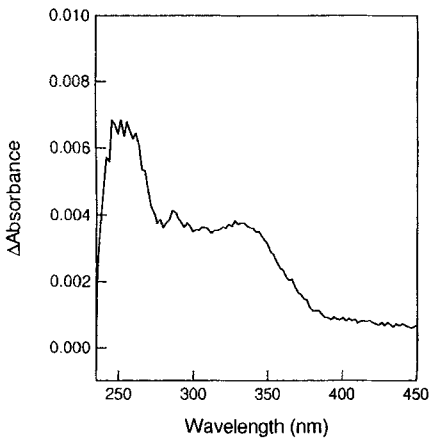

Fig. 7. Effect of activator protein on the absorption spectrum of MDH. Difference absorption spectrum of MDH, recorded after addition of 5 mM Mg^{2+} to a mixture consisting of MDH (6.5 μM), activator protein (200 nM), and NAD (25 μM) in buffer A. The spectral change was observed only in the presence of activator protein, NAD, as well as Mg^{2+}.

DISCUSSION

Spectral analysis of purified MDH (Fig. 1) revealed the presence of a chromophore with redox activity towards the MDH substrates formaldehyde and methanol (Fig. 2). Apparently, the chromophore participates in the alcohol dehydrogenase and aldehyde reductase reactions catalyzed by MDH, thus serving as a cofactor. The cofactor could be dissociated by protein denaturation, which indicates a tight, but non-covalent, interaction with MDH. The extracted cofactor was identified as NADH by ion-exchange chromatography (Fig. 4) and by its biological activity in standard horse liver ADH assays. The MDH absorption spectrum (Fig. 1) is similar to that of other known NAD(P)(H)-containing enzymes (Kato et al., 1983, 1986; Zachariou and Scopes, 1986). The shoulder around 320 nm displayed by these enzymes was reported to be due to the reduced form of the cofactor. Quantitative studies revealed that MDH most likely contains 1 molecule NAD(H) per subunit.

Incubations of MDH with [^{32}P]NAD resulted in labelling of only 6–10% MDH subunits. A much higher labelling intensity was expected if exchange of MDH-bound NAD(H) and exogenous [^{32}P]NAD occurred as an obligate step in the MDH catalytic cycle. The incorporation of label was virtually independent of the presence of methanol (or activator protein), i.e. it even occurred without catalytic activity (Table 1). The observed incorporation of label in MDH protein thus may simply represent direct binding of [^{32}P]NAD to available empty binding sites (0.42–0.95 mol NADH/mol subunit was detected in the MDH preparations used in these studies; see above). MDH strictly required (catalytic amounts of) exogenous NAD for methanol-dependent 2,6-dichloroindophenol reduction in the presence of phenazine methosulfate. Hence, NAD(H) appears to have two different roles, with MDH-bound NAD (cofactor) serving as the primary electron acceptor in the alcohol dehydrogenase reaction, and exogenous NAD (coenzyme) being responsible for the reoxidation of MDH-bound NADH. Further support for this comes from the observation that methanol oxidation by MDH from *B. methanolicus* follows a ping-pong type mechanism (Fig. 5). Such a mechanism corresponds to a catalytic cycle in which the first product is released from the enzyme before binding of the second substrate. In this respect, MDH resembles glucose-fructose oxidoreductase from *Z. mobilis* (Zachariou and Scopes, 1986) and formaldehyde dismutase from *Pseudomonas putida* F61 (Kato et al., 1983, 1986). MDH and these two oxidoreductases display ping-pong mechanisms because a bound NAD(P)(H) serves as a temporary deposit of reducing equivalents. In contrast, commonly observed NAD-dependent alcohol dehydrogenases, lacking a bound NAD(P)(H), such as horse liver ADH, generally obey a sequential reaction mechanism that proceeds via a ternary enzyme · substrate · nucleotide complex (Sekhar and Plapp, 1990). It was demonstrated that horse liver ADH protein provided with a NAD analogue covalently bound in its active site also displayed a ping-pong type mechanism (Kovář et al., 1984).

Previously, we reported the absence of the proposed fingerprint of a $\beta\alpha\beta$-dinucleotide-binding fold (Wierenga et al., 1986) in the MDH sequence (De Vries et al., 1992). The specific interactions between MDH, its tightly bound NAD(H) cofactor, and exogenous NAD, and the identity and properties of the NAD-binding site(s) involved are currently investigated in more detail. Each of the subunits of the activator protein (this study) and MDH protein (Vonck et al., 1991) also contains 1 Zn^{2+} and 1–2 Mg^{2+} ion(s). As reported, the presence of exogenous Mg^{2+} ions is essential for stimulation of MDH activity by the activator protein (Arfman et al., 1991). In the present study we observed that Mg^{2+} ions are essential for the formation of a MDH · activator protein · NAD complex (Figs 6 and 7). Further work is required to establish the precise role of these metal ions.

To identify the step in the MDH reaction cycle that is stimulated by the activator protein, we probed for the rate-limiting step. Starting with purified MDH, containing bound cofactor NADH (MDH_{red}), the following five steps can be distinguished in its reaction cycle (Fig. 8): binding of exogenous NAD to MDH_{red} (step 1), transfer of reducing equivalents from the NADH cofactor to the NAD coenzyme (step 2), binding of methanol and release of NADH coenzyme (step 3), oxidation of methanol by the MDH-bound NAD cofactor (step 4), and formaldehyde release (step 5). The absence of a significant deuterium isotope effect in both activator (in)dependent reactions, clearly indicated that the MDH reaction rate is controlled by a step other than one involving C-H bond breaking, e.g. hydride transfer (step 4). Since purified MDH contains no formaldehyde, release of formaldehyde also appears not to be rate-limiting (step 5). The most likely steps therefore are reoxidation of MDH_{red}

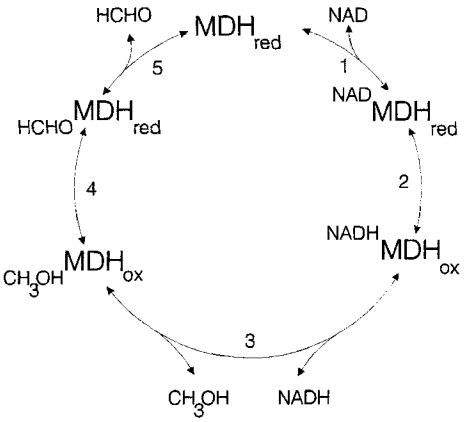

Fig. 8. Reaction cycle of MDH. See text for details of numbered steps.

(step 2) or release of coenzyme NADH from MDH (step 3). NADH-binding studies showed that the activator protein is able to bind NADH (see above). The presence of the NAD(H) chromophore in MDH enabled us to demonstrate the actual formation of a MDH · activator protein complex in the presence of NAD and Mg^{2+} (Figs 5, 6 and 7). This complex formation could not be demonstrated by standard biochemical methods (Arfman et al., 1991) and may be relatively easily dissociable. These observations suggest that the activator protein facilitates oxidation of the reduced NADH cofactor of MDH (step 2).

MDH from *B. methanolicus* is the first example of a naturally occurring NAD-dependent alcohol dehydrogenase containing tightly bound NAD(H). Examples of other bacterial enzymes possessing a tightly bound NAD(P)(H) are as follows: formaldehyde dismutases from *P. putida* F61 (Kato et al., 1983, 1986), *A. methanolica*, and *M. gastri* (Bystrykh et al., 1993a–c), glucose-fructose oxidoreductase from *Z. mobilis* (Zacharniou and Scopes, 1986) and malate-lactate transhydrogenase from *Micrococcus lactilyticus* (Allen, 1966). In contrast to these five oxidoreductases, *B. methanolicus* MDH requires exogenous NAD as electron acceptor for activity. Formaldehyde dismutase can use formaldehyde both as a donor and acceptor of reducing equivalents, which results in formation of methanol and formate (Kato et al., 1986; Bystrykh et al., 1993b). Formaldehyde dismutase enzymes are conveniently assayed by following the methanol-dependent reduction of the aldehyde analog *p*-nitroso-*N*,*N*-dimethylaniline (Dunn and Bernhard, 1971). MDH did not possess alcohol:*p*-nitroso-*N*,*N*-dimethylaniline oxidoreductase activity, again clearly differing from these dismutase enzymes.

NAD-dependent alcohol dehydrogenases are found in many organisms and are capable of oxidizing a broad range of primary and secondary alcohols. In general, their affinity for methanol is very poor (Brändén et al., 1975). For example, for the horse liver ADH and *Bacillus stearothermophilus* ADH enzymes K_m values for methanol of 30 mM and 20 mM, respectively, have been determined (Sheehan et al., 1988). Whole cells of *B. methanolicus* displayed an affinity constant (K_s) for methanol of 2.6 mM (Arfman et al., 1989). Previous studies with purified NAD-dependent MDH of *B. methanolicus* showed that the activator protein significantly increases the affinity and specific activity of MDH toward ethanol and methanol as a substrate (Arfman et al., 1991). Kinetic data showed that at physiological methanol concentrations (0.1–1 mM), the methanol turnover rate of MDH is increased up to 40-fold by the activator protein. The unusual structural and mechanistic properties of MDH present in methylotrophic bacilli, as well as the involvement of an activator protein, suggest that this combined enzyme system is

highly specialized towards oxidation of short-chain primary alcohols, including methanol.

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